DEVELOPMENTAL BIOLOGY 176, 17–21 (1996)
ARTICLE NO. 0111

Early Embryonic Induction in *C. elegans* Can Be Inhibited with Polysulfated Hydrocarbon Dyes

Olaf Bossinger and Einhard Schierenberg
Zoologisches Institut, Universität Köln, Kerpener Strasse 15 50923 Cologne, Germany

During embryogenesis of *Caenorhabditis elegans* cellular interactions are necessary to determine the fate of blastomeres. In one of these interactions, taking place in the 4-cell stage, the germline cell P2 induces longitudinal orientation of the cleavage spindle in the neighboring EMS cell, its asymmetric division, and the establishment of a gut lineage. Application of several polysulfated hydrocarbon dyes (e.g., trypan blue, TB) in the 1- to 4-cell stages inhibits induction of the gut precursor cell. However, dye application from the late 4-cell stage onward does not interfere with gut induction, supporting the earlier finding of a short time window for this interaction. We also tested the effect of TB on the induction of pharyngeal muscle cells by the MS blastomere, which appears to involve a surface receptor–ligand interaction. We found that this process is inhibited as well. These and additional data indicate that polysulfated hydrocarbon dyes are suitable tools to generally interfere with cell–cell interactions in the nematode embryo.

INTRODUCTION

During early embryogenesis of *Caenorhabditis elegans* several inductive interactions have been identified (Bowerman, 1995). One of these affects the establishment of the gut lineage. The founder cell of the gut (E-cell) is established in the 6-cell stage with the asymmetric division of its mother cell EMS (Fig. 1a). This asymmetric division is induced by the germline cell P2 between the 4- and 6-cell stages, which can be demonstrated by blastomere isolation and recombination experiments (Schierenberg, 1987; Goldstein, 1992). The basis of the inductive interaction between P2 and EMS is unknown.

Another interaction takes place between the 8- and 12-cell stages when descendants of the MS cell induce anterior AB cells to produce pharyngeal muscle cells (Priess and Thomson, 1987; Hutter and Schnabel, 1994). Posterior AB descendants are not competent to react to the inducing MS signal because of an earlier inhibitive interaction between P2 and ABp (Mello *et al.*, 1994). There is good evidence that these interactions involve a cell surface receptor/ligand mechanism (Evans *et al.*, 1994).

Polysulfated hydrocarbon dyes (PHDs) bind strongly to the plasma membrane. Due to their negative charges a mechanism has been postulated through which they bind to positively charged side chains of surface molecules, thus inhibiting the coupling of receptor and ligand (Röhrkasten and Fehrenz, 1987). Here we show that several early inductions in the *C. elegans* embryo can be inhibited with PHDs.

MATERIALS AND METHODS

C. elegans was maintained essentially as described by Brenner (1974). Eggs were dissected out of gravid adults with a scalpel in distilled water and transferred with a drawn-out mouth pipette to a microscope slide, where they were stuck to a thin polylysine layer.

Laser penetration was performed in cell culture media containing 15% fetal calf serum (FCS) (Bossinger and Schierenberg, 1992; Edgar, 1995, modified) with one of three tested PHDs in the following concentrations: 14 mg/ml trypan blue (Mr 961; Sigma), 6 mg/ml Evans blue (Mr 961; Serva), 4 mg/ml Chicago sky blue (Mr 993; Sigma). These concentrations reliably block interactions without showing toxic effects. With a N_2-pumped dye laser (Lambda Physics, Göttingen) coupled to a microscope the eggshell and underlying vitelline membrane were perforated with brief pulses using the laser dye rhodamine 6G. To neutralize the inhibitive effect of TB in pulse experiments, 15% FCS was replaced by 40% FCS. High concentrations of calf serum appear to remove TB from the cell surface indicated by the disappearance of fluorescent marking.

Development of embryos was observed with Nomarski optics and epiillumination (100-W mercury bulb) to visualize autofluorescence (excitation 340–380 nm) and PHD-induced fluorescence (excitation 520–560 nm) using a Leica DM IRBE inverted microscope equipped with a Leica 100× PL Fluotar oil-immersion objective (NA 1.3). Tissue differentiation was visualized with monoclonal antibodies (Okamoto and Thomson, 1985) ICB4 (gut) and 3NB12 (pharynx) after standard methanol–acetone fixation (Strome and Wood, 1983). Manipulated embryos were documented as described in Bossinger and Schierenberg (1992).

0012-1606/96 $18.00

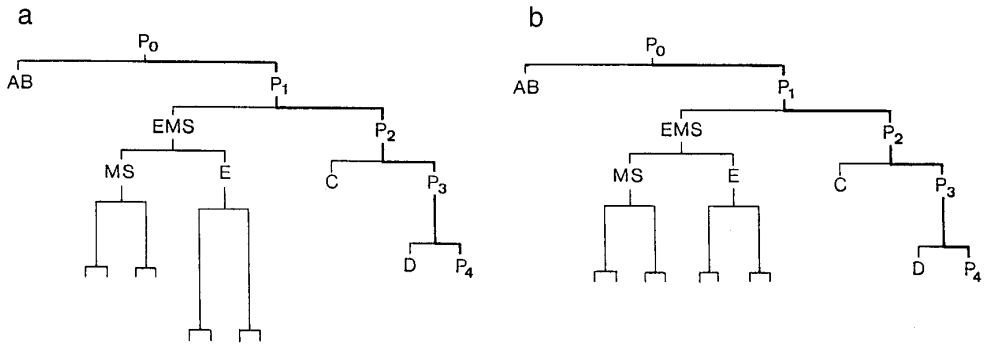

FIG. 1. Early lineage trees of *C. elegans*. In a series of asymmetric germline divisions (P0–P3) five somatic founder cells (AB, MS, E, C, and D) and the primordial germ cell P4 are generated. (a) Under normal conditions the E cell enters mitosis slightly after MS but the descendants of E express considerably longer cell cycle periods than those of MS. (b) After application of trypan blue, MS and E and their descendants cleave synchronously. Both lineages are drawn with the same time scale.

RESULTS AND DISCUSSION

Polysulfated Hydrocarbon Dyes Cause Transverse Spindle Orientation in EMS and Change Cell Cycle Timing

We investigated the influence of three different PHDs on cellular interactions between EMS and P2. Embryos in the 1- to early 4-cell stages were perforated in a cell culture medium containing trypan blue, Evans blue, or Chicago sky blue. The dyes bind strongly to cell membranes and mark their contours under fluorescent light (Fig. 2a). Under these conditions the EMS cell divides with a transverse (Figs. 2c and 2d; $n > 20$) instead of a longitudinal spindle orientation (Fig. 2b). Consequently, the cell cycle rhythms of both EMS descendants instead of becoming different (Fig. 1a) remain the same during the subsequent divisions (Figs. 1b, 2e and 2f). Controls in which 1- to 4-cell embryos had been preincubated with dye (to allow absorption of the laser beam) and then penetrated in medium without dye always established a proper gut lineage ($n = 8$).

PHDs Inhibit Gut Differentiation

The incubation of *C. elegans* embryos with PHDs as described above results in a terminal phenotype with cell numbers similar to those of a hatching worm but without visible initiation of outer morphogenesis. Such embryos show several signs of cell differentiation, e.g., programmed cell death and muscle twitching, which indicates that the dyes in the applied concentrations are not generally toxic (Fig. 2g). However, no signs of gut differentiation were found. We looked for three markers, which are characteristic for differentiated gut cells in untreated embryos: autofluorescence (Bossinger and Schierenberg, 1992), birefringent cytoplasmic granules (Laufer *et al.*, 1980), and binding of the gut-specific antibody ICB4. In the manipulated embryos we detected neither birefringence ($n = 12$; Fig. 2h) nor antibody binding ($n = 12$; data not shown) under our experimental conditions. However, some autofluorescence developed all over the embryo (Fig. 2i). Together with similar findings after inhibition of gut-specific endocytosis (Bossinger and Schierenberg, 1996), this suggests that autofluorescence is not an exclusive product of the intestine itself but involves intercellular transfer processes (Bossinger and Schierenberg, 1992).

Trypan Blue Affects Spindle Orientation and Gut Induction

Recently Goldstein (1995a) showed that during the interaction between P2 and EMS two phases can be discerned, one which assures proper spindle orientation and a second which is sufficient for induction. To test whether we can affect both or only one of these phases, we added TB at various times of the EMS cell cycle.

Our data (Table 1) show that both aspects of interaction can be affected. From the 1-cell stage onward to Minute 4 of the EMS cell cycle TB always leads to transverse spindle orientation. We found that this abnormality is incompatible with gut formation consistent with the observation by Goldstein (1995a). Later application of TB does not lead to transverse spindle orientation but gut differentiation is still suppressed up to about Minute 7. From about Minute 9 onward TB no longer interferes with the establishment of the gut lineage: embryos develop the typical birefringent and autofluorescent granules in the area where the descendants of the E-cell are localized (Figs. 2k and 2l).

Even if proper gut induction had taken place, embryos do not hatch but develop to a terminal phenotype without proper morphogenesis (Fig. 2j). This may indicate that TB interferes with additional embryonic cell–cell interactions.

TB Also Interferes with Pharyngeal Induction

To test whether the inhibition described above is specific to gut induction we studied the influence of TB on induc-

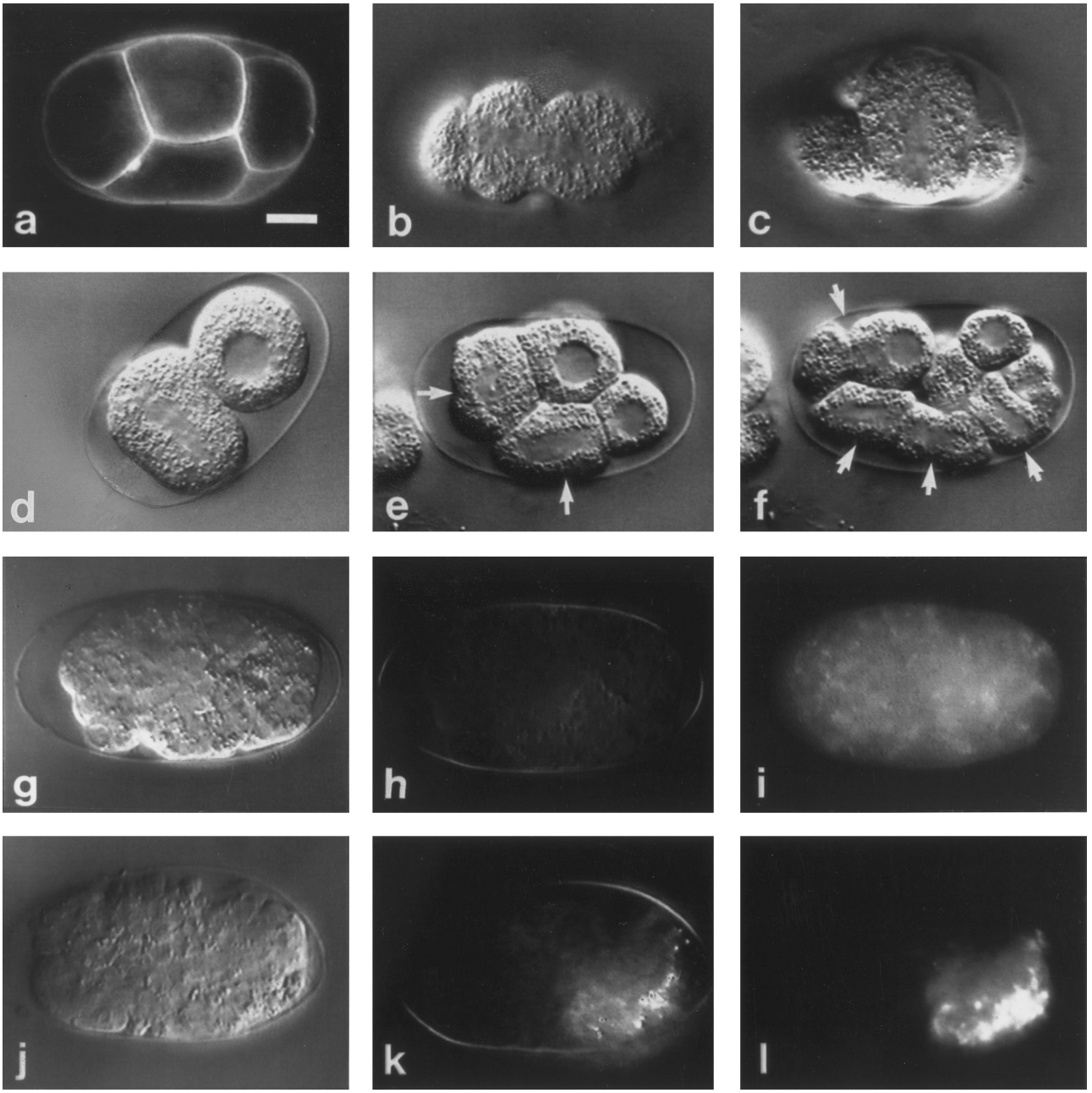

FIG. 2. Incubation of early embryos with trypan blue changes mitotic spindle orientation of EMS, changes the division rhythm of both daughters, and inhibits gut differentiation. (a) 4-cell stage; after laser penetration in TB-containing medium, the medium was exchanged with TB-free medium to remove background fluorescence. (b) Untreated control embryos show an anterior–posterior spindle orientation in the EMS cell. (c) TB-treated embryos show a transverse spindle orientation. (d) After incubation with TB from the 1- to 4-cell stages onward, EMS divides transversely. Both EMS daughters (e; arrows) and all four EMS granddaughters (f; arrows) divide synchronously. For better visibility the AB cell has been removed. After incubation with TB from the 1- to 4-cell stage onward, the terminal phenotype (g) expresses no birefringence (h) but a bluish autofluorescence in all cells (i). After incubation with TB from the late 4-cell stage onward the terminal phenotype (j) shows a localized appearance of birefringent (k) and autofluorescent granules (l). Nomarski optics (b–g, j); epifluorescence, excitation 520–560, (a); epifluorescence, excitation 340–380, (i,l); polarization optics (i,l). Orientation: a,d–l, lateral view; anterior, left. b, lateral view; anterior, right. c, ventral view; anterior, left. Bar, 10 μm.

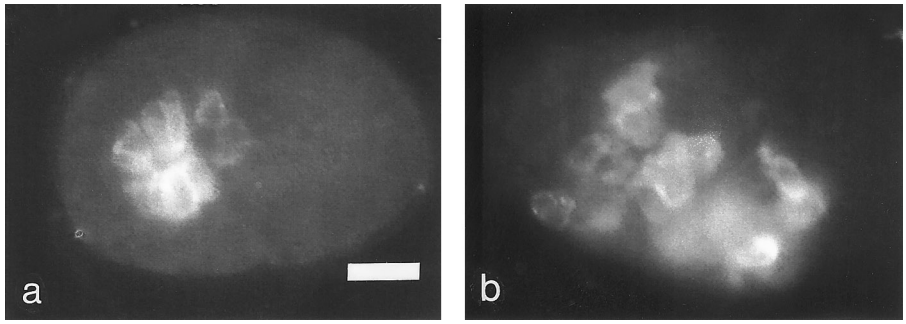

FIG. 3. Immunofluorescence staining of pharyngeal muscle cells in *C. elegans* embryos after trypan blue (TB) treatment. (a) Control embryo (end of proliferation phase) with 21 pharyngeal muscle cells. (b) Production of extra pharyngeal cells in an embryo treated with a pulse of TB at the 4-cell stage. Approximately 35 cells were counted in this embryo. As stained cells are in different focal planes, not all of them are visible. Orientation: anterior, left. Bar, 10 μm.

tion of pharyngeal cells (see Introduction). For this we performed two experiments. In the first of these we added TB to embryos at the early 8-cell stage. This is too late to prevent gut induction by P2 but early enough to suppress induction of anterior AB cells by MS to generate pharyngeal muscle cells. After development to the terminal phenotype (after 15–24 hr) the presence of pharyngeal muscle cells was examined with the antibody 3NB12. In untreated embryos 21 pharyngeal cells (14 from MS and 7 from AB) are stained with this antibody (Priess and Thomson, 1987; Hutter and Schnabel, 1994). In some cases exact counting was difficult as several stained blastomeres were positioned on top of each other. We counted 12 ($\pm$2) cells in a single cluster; n = 6), indicating the absence of AB-derived pharynx muscle cells. In control experiments (n = 4), when TB was added after the 12-cell embryo, we counted 18 ($\pm$3) cells (a larger plus a smaller cluster). This result, indicating that MS induces AB descendants in the 8- to 12-cell embryo, is in accordance with the conclusions drawn by Hutter and Schnabel (1994).

In a second experiment we took advantage of our finding that TB can be washed out (see Materials and Methods) and thus inductive interactions can be inhibited during preselected periods. In a first step we added TB between the 1- and 4-cell stages. This should prevent (i) the inhibition of the posterior AB cell (ABp) by P2 to become competent to the inductive signal of MS (Mello *et al.*, 1994; Hutter and Schnabel, 1994; Mango *et al.*, 1994) and (ii) cause EMS to generate two MS-like daughters (Goldstein, 1995b). In the 7-cell stage we washed out TB to allow inductive interactions between MS and AB cells to generate pharyngeal muscles. Under these conditions we counted nearly twice the normal number of pharyngeal muscle cells (36 $\pm$ 4; n = 4). This number and the position of stained cells (Fig. 3) suggests that both EMS daughters and in addition, ABa and ABp descendants had produced pharyngeal cells. This production of ectopic pharyngeal cells is consistent with a block in both of the interactions described above, and suggests that subsequent development including later cell–cell interactions and tissue differentiation are largely unaffected in embryos treated with a pulse of TB at the 4-cell stage.

Our recent results show that the tested dyes also affect other kinds of cell–cell communication (e.g., endocytosis; Bossinger and Schierenberg, 1996). Thus, they not only seem suitable to interfere with various cellular interactions in the nematode embryo but in addition allow blocking of selected inductions due to the reversibility of the inhibitory effect.

TABLE 1
Behavior of the EMS Cell after Application of Trypan Blue

	Time of application (min of EMS cell cycle)[a]			
	4	6	8	10
Spindle orientation (T, transverse; O, oblique)	T	O	O	O
Gut differentiation	0/5	0/6	3/6	10/10

[a] Measured from beginning of cytokinesis.

ACKNOWLEDGMENTS

We are obliged to Renate Seiler for photography, Elisabeth Trojtza for technical assistance, Oliver Wiegner for help with experiments, and Randy Cassada for advice. This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

Bossinger, O., and Schierenberg, E. (1992). Transfer and tissue-specific accumulation of cytoplasmic components in embryos of

Caenorhabditis elegans and *Rhabditis dolichura:* In vivo analysis with a low-cost signal enhancement device. *Development* 114, 317–330.

Bossinger O., and Schierenberg E. (1996). Embryonic gut differentiation in nematodes: Endocytosis of macromolecules and its experimental inhibition. *Roux's Arch. Dev. Biol.*, in press.

Bowerman, B. (1995). Determinants of blastomere identity in the early *C. elegans* embryo. *Bioessays* 17, 405–414.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans. Genetics* 77, 71–94.

Edgar, L. G. (1995). Blastomere culture and analysis. *In Caenorhabditis elegans:* Modern Biological Analysis of an Organism. (H. F. Epstein and D. C. Shakes, Eds.), Methods in Cell Biology, Vol. 48, pp. 303–321. Academic Press, San Diego.

Evans, T. C., Crittenden, S. L., Kodoyianni, V., and Kimble, J. (1994). Translational control of maternal *glp-1* mRNA establishes an asymmetry in the *C. elegans* embryo. *Cell* 77, 183–194.

Goldstein, B. (1992). Induction of gut in *Caenorhabditis elegans* embryos. *Nature* 357, 255–257.

Goldstein, B. (1995a). Cell contacts orient some cell division axes in the *Caenorhabditis elegans* embryo. *J. Cell Biol.* 129, 1071–1080.

Goldstein, B. (1995b). An analysis of the response to gut induction in the *C. elegans* embryo. *Development* 121, 1227–1236.

Hutter, H., and Schnabel, R. (1994). *glp-1* and inductions establishing embryonic axes in *C. elegans. Development* 120, 2051–2064.

Laufer, J. S., Bazzicalupo, P., and Wood, W. B. (1980). Segregation of developmental potential in early embryos of *Caenorhabditis elegans. Cell* 19, 569–577.

Mango, S. E., Thorpe, C. J., Martin, P. R., Chamberlain, S. H., and Bowerman, B. (1994). Two maternal genes, *apx-1* and *pie-1,* are required to distinguish the fate of equivalent blastomeres in the *Caenorhabditis elegans* embryo. *Development* 120, 2305–2315.

Mello, C. C., Draper, B. W., and Priess, J. R. (1994). The maternal genes *apx-1* and *glp-1* and the establishment of dorsal–ventral polarity in the early *C. elegans* embryo. *Cell* 77, 95–106.

Okamoto, H., and Thomson, J. N. (1985). Monoclonal antibodies which distinguish certain classes of neuronal and supporting cells in the nervous tissue of the nematode *Caenorhabditis elegans. J. Neurosci.* 5, 643–653.

Priess, J. R., and Thomson, J. N. (1987). Cellular interactions in early *C. elegans* embryos. *Cell* 48, 241–250.

Röhrkasten, A., and Ferenz, H. J. (1987). Inhibition of yolk formation in locust oocytes by trypan blue and suramin. *Roux's Arch. Dev. Biol.* 196, 165–168.

Schierenberg, E. (1987). Reversal of cellular polarity and early cell–cell interaction in the embryo of *Caenorhabditis elegans. Dev. Biol.* 122, 452–463.

Strome, S., and Wood, W. B. (1983). Generation of asymmetry and segregation germ-line granules in early *C. elegans* embryos. *Cell* 35, 15–25.

Received for publication January 24, 1996
Accepted February 7, 1996